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Sequence and Tissue Distribution of the Integrin α9 Subunit, a Novel Partner of β1 That Is Widely Distributed in Epithelia and Muscle

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Abstract. The integrin family of adhesion receptors consists of several heterodimeric glycoproteins, each composed of one α and one β subunit. A novel integrin α subunit partial cDNA isolated from TGF-β stimulated guinea pig airway epithelial cells has previously been reported (Erle, D. J., D. Sheppard, J. Bruess, C. Rüegg, and R. Pytela. 1991. *Am. J. Respir. Cell Mol. Biol.* 5:170–177). We have now determined cDNA and amino acid sequence for the human homolog of this subunit, named α9, from a human lung cDNA library, a human small intestine cDNA library, and cDNA from the cell lines U937, HL-60 and Tera-2. This sequence is predicted to encode a 1006-amino acid mature protein that shares 39% identity with the previously identified integrin subunit α4. By Northern blot analysis, α9 mRNA was detected in the human carcinoma cell lines Tera-2 and Caco-2. Anti-peptide antibodies against the predicted COOH-terminal sequence of α9 immunoprecipitated a heterodimer (140 kD/115 kD nonreduced; 150 kD/130 kD reduced) from Tera-2 lysates. Immunodepletion of β1-containing integrins with Tera-2 lysates removed α9 immunoreactivity, suggesting that β1 is the principal β subunit partner for α9 in these cells. α9 was detected by immunohistochemistry in airway epithelium, in the basal layer of squamous epithelium, and in smooth muscle, skeletal muscle, and hepatocytes.

THE integrins are a large family of cell surface glycoproteins that mediate cell–cell and cell–matrix adhesion (21). All known members of this family are heterodimers consisting of an α and a β subunit that bind noncovalently to each other. Published reports suggest the existence of 8 β subunits (β1–β8) (2, 12, 14, 20, 26, 30, 32, 34, 37, 40, 41) and 16 α subunits (2–5, 9, 10, 13, 16, 23, 25, 28, 33, 38, 39, 42–47). 13 of these have been completely sequenced (α1–α8, αv, αM, αL, αX, αIIb). The existence of αIEL and αLRI has been established on the protein level. We have previously reported identification of partial integrin α subunit cDNA sequences using degenerate oligonucleotide primer pairs and the PCR (13). Primers were designed based on two highly conserved regions, separated by 72–92-amino acid residues, located within the fifth and sixth repeated domains of the previously reported integrin α subunits. PCR amplification of cDNA using these primers resulted in the identification of several integrin α subunit partial cDNAs. From guinea pig airway epithelial cells six different sequences were identified. Five of these were 88 to 92% identical to the sequences of α1–α3, α5 and αv from other species and were presumed to encode the guinea pig homologs of those subunits. One of the amplified sequences was only 24–60% identical to previously reported α subunits. This novel cDNA was predicted to encode a 71-amino acid fragment of an integrin α subunit, provisionally designated αA. We now report the predicted coding sequence of the human homolog of this novel subunit. We have identified mRNA encoding this subunit and the corresponding protein in two cell lines, including the human teratoma cell line Tera-2, and demonstrate that this α subunit associates principally with the integrin β1 subunit in these cells. Based on the convention of sequentially numbering newly identified partners of β1, we have called this α subunit α9. We also demonstrate wide distribution of α9 in epithelia, smooth muscle, skeletal muscle, and hepatocytes.

Materials and Methods

Cell Lines, RNA Purification, and cDNA Synthesis

ATCC human cell lines were maintained in DME (HeLa, Tera-2, Caco-2,) or RPMI 1640 media (U937, HL-60, Raji, HuT 78, MOLT-4) containing 10% FBS. Media were obtained from BioWhittaker, Inc. (Walkersville, MD).

mRNA was isolated from resected human lung tissue using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Total cellular RNA

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was isolated using the LiCl/Urea method (12). Single-stranded cDNA was synthesized at 44°C for 1 h from 1 to 5 μg of mRNA or 20–40 μg of total RNA using the Superscript cDNA Synthesis System (GIBCO-BRL, Gaithersburg, MD) and random DNA hexamers. In some cases cDNA was synthesized as above using a previously described sequence tagged random DNA hexamer primer (CGAGGGGGATGGTCGACGGAAGCGACC-NNNNNNT) (15).

Amplification and Purification of Lambda Library DNA

Aliquots of λgt11 libraries containing 1–5 million plaque forming units were mixed with 500 μl of LE392 bacteria (OD = 0.5) in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.0, 0.01% gelatin), incubated 15 min at 37°C, and shaken at 250 rpm overnight at 37°C in Luria broth containing 10 mM $MgCl_2$ and 0.2% maltose. The cultures were incubated at 37°C and 250 rpm for 30 min with 5% chloroform. Aliquots of the aqueous layer were subjected to three cycles of 3 min in dry ice/ethanol followed by 3 min at 90°C, phenol/chloroform extraction, and precipitation with ethanol.

PCR Amplification

PCR reactions were performed in 25–200 μl reaction volumes and contained 1 × *Taq* buffer (Promega, Corp., Madison, WI) with 1.5 mM $MgCl_2$, 0.1 μM each of two primers, 0.025 U/μl *Taq* Polymerase (Promega Corp.), 1–8 μl of DNA template, and 0.1 mM each of dATP, dGTP, dCTP, and dTTP. Reactions were heated to 94°C for 4 min in a thermocycler (ERICOMP, Inc., San Diego, CA) and then subjected to 30 cycles of PCR followed by 10 min at 72°C. Reactions that contained degenerate oligonucleotide primers were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 48 or 53°C, and 45 s at 72°C. Reactions that contained α9-specific primer pairs designed to amplify DNA fragments less than 750 nucleotides were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 57°C, and 60 s at 72°C. Reactions that contained an α9-specific primer, a λgt11-specific primer (λgt11F: CCTCCGTCGACGGTGGCGACGACTCCTGGAGCCCG or λgt11R: CCTCCGTCGACTTGACACCAGACCAACTGGTAATG), and purified concentrated total library DNA were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 60°C, and 120 s at 72°C. All other PCR reactions were subjected to cycles consisting of 45 s at 94°C, 45 s at 57°C, and 120 s at 72°C. Products of each PCR reaction were analyzed by agarose gel electrophoresis using standard or low gel temperature agarose.

PCR reactions designed to amplify 3' sequence from sequence tagged cDNA, described above, contained α9-specific forward primers and primers complementary to the sequence tagged random hexamer (GATGGTCGACGGAAGCGAAC or CGAGGGGGATGGTCGACGG) and were performed as above.

Cloning of DNA Fragments

Restriction-digested DNA fragments were isolated on low gel temperature agarose and purified by phenol/chloroform extraction and ethanol precipitation. Fragments were ligated into restriction-digested, dephosphorylated pBluescript vector (Stratagene, La Jolla, CA) with T4 DNA ligase (GIBCO-BRL). The ligation mixture was used to transform competent *Escherichia coli* (JM-109; Clontech, Palo Alto, CA). Selected plasmids were purified from liquid cultures using the Pharmacia miniprep lysis kit (Pharmacia Fine Chemicals/LKB, Pleasant Hill, CA) and sequenced using Sequenase 2.0 (United States Biochemical Corporation, Cleveland, OH), ^{35}S-dATP (Amersham Corp., Arlington Heights, IL), T3 primer, T7 primer, and a variety of primers specific for α9.

Library Screening

Partial cDNA fragments were used as templates for the random-primed synthesis of [^{32}P]dCTP-labeled probes (multiprime DNA labeling system; Amersham Corp.). These probes were used to screen an oligo-dT-primed λgt11 cDNA library from human lung tissue including trachea and bronchioles (catalog No. HL 1066b; Clontech) and a mixed random and oligo-dT primed λgt11 cDNA library from human small intestine tissue (catalog No. HL1133b; Clontech). Hybridizations were performed at 50°C for 16 h in hybridization buffer (40% formamide, 50 mM sodium phosphate, pH 6.5, 800 mM NaCl, 0.05% polyvinylpyrrolidone, 0.05% BSA, 0.05% ficoll, 1 mM EDTA, 0.1% SDS, and 10 ng/ml heat-denatured, sonicated salmon sperm DNA). Filters were washed twice in 1× SSC containing 0.1% SDS for 5 min at room temperature, and once in 0.5× SSC containing 0.1% SDS for 1 h at 50°C and exposed to film for 18 h at −80°C with intensifying screens. Positive library clones were isolated by further rounds of screening, and the inserts were isolated either by PCR amplification using λgt11-specific primers or by EcoRI digestion of purified phage DNA. The inserts were then subcloned into EcoRI-digested pBluescript and sequenced.

Northern Blot Analysis

Total cellular RNA was electrophoresed through a formamide–agarose gel and transferred to a nylon membrane (Hybond-N; Amersham Corp.). Radiolabeled probe was synthesized from 2.1 kb of α9 sequence using the Multiprime Labeling System (Amersham Corp.) and [^{32}P]dCTP. Filters were incubated at 50°C for 16 h in hybridization buffer containing 5× SSC, 40% formamide, 20 mM Tris, pH 7.5, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.1% ficoll, 10% dextran sulfate, and 100 μg/ml heat-denatured, sonicated salmon sperm DNA, and washed in 5× SSC containing 0.1% SDS at 50°C for 30 min. After washing, filters were exposed to film at −80°C with an intensifying screen.

Antibodies and Immunoprecipitations

Polyclonal antiserum was generated against the α9 peptide CRKENEDSW-DWVQKQ. Peptide synthesis, conjugation to KLH, and injection of rabbits was performed by Immunodynamics, Inc. (La Jolla, CA). α9 antibodies were affinity purified from crude antiserum on a peptide-lysozyme-Affigel column as follows. Affigel-10 slurry (4-ml bed volume) (Bio Rad Labs., Richmond, CA) was washed three times with cold 10 mM sodium acetate, pH 4.5; rinsed once with 0.1 M potassium phosphate, pH 7.5; mixed with 100 mg lysozyme (Sigma Immunochemicals, St. Louis, MO) in 8 ml 0.1 M potassium phosphate, pH 7.5, for 2 h at room temperature; washed twice with 0.1 M potassium phosphate, pH 7.5; mixed with 8 ml 0.2 M ethanolamine, pH 8.0, for 2 h at room temperature; and washed three times with PBS. Lysozyme-Affigel (600-μl bed volume) was washed with 0.05 M sodium phosphate, pH 8.0; mixed with 2.5 mg sulfo-*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemicals, Rockford, IL) in 1 ml 0.05 M sodium phosphate, pH 8.0, for 30 min at room temperature; washed with 0.05 M sodium phosphate; pH 8.0; mixed with 3 mg α9 peptide in 500 μl 0.05 M sodium phosphate, pH 8.0, for 3 h at room temperature; and washed with (*a*) 0.05 M sodium phosphate, pH 7.0, (*b*) 200 mM glycine, pH 1.5, (*c*) 0.05 M sodium phosphate, pH 7.0, (*d*) 8 M urea, and finally (*e*) 0.05 M sodium phosphate, pH 7.0. 5–10-ml aliquots of antiserum were mixed with the peptide-lysozyme-Affigel overnight at 4°C. The slurry was transferred to a column and washed with 0.05 M sodium phosphate, pH 7.0, until the OD 280 of the wash buffer was less than 0.01. Antibodies were eluted in 1.25-ml fractions with 200 mM glycine, pH 1.5, and collected in tubes containing 250 μl 1 M sodium phosphate, pH 8.0. Pooled fractions were dialyzed overnight at 4°C against PBS containing 0.02% azide.

mAb P5D2 (11) directed against the β1 integrin subunit was a gift from Elizabeth Wayner (University of Minnesota, Minneapolis, MN). mAb R6G9 directed against the β6 integrin subunit was generated in our laboratory. For some experiments antibodies were cross linked to protein A-Sepharose with dimethylpimelimidate (17).

Cells were surface labeled with [^{125}I], lysed in immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 0.1% NP-40, and 300 mM NaCl), and immunoprecipitated by standard techniques. Samples were analyzed by SDS-PAGE on 7.5% acrylamide gels and exposed to film at −80°C with intensifying screens.

Tissue Staining

Frozen sections (5 μm) of tissue were fixed in either 2% paraformaldehyde (mouse tissue) at room temperature or in −18°C acetone (human tissue) for 5 min and subsequently rinsed in PBS. Sections were blocked for endogenous peroxidase activity with Peroxoblock Solution (Zymed Laboratories, Inc., South San Francisco, CA) for 45 s at room temperature. After rinsing, sections were preblocked with 0.5% casein/0.05% thimerosal/PBS for 15 min at room temperature and then incubated overnight at 4°C in primary antibody (1:200 in 0.5% casein/0.05% thimerosal/PBS) that either was or was not preincubated with 0.1 mg/ml α9 peptide for 30 min at 4°C. After rinsing in PBS, sections were incubated in either biotinylated donkey anti-rabbit secondary antibody (Amersham Corp.) at a dilution of 1:200 or peroxidase-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:250 for 1 h at room temperature in 0.5% casein/0.05% thimerosal/PBS. Sections incubated with biotinylated secondary antibodies were then rinsed and incubated in ABC avidin/peroxidase reagent (Vector Laboratories) for 1 h at room temperature. Chromagen

was developed using the DAB Plus Kit from Zymed Laboratories. Reactions were monitored until suitable color development was achieved. The signal was enhanced with 0.5% nickel chloride, and the sections were rinsed in distilled water. Sections were subsequently air dried and then mounted with Permount (Fisher Scientific, Pittsburgh, PA) onto clean slides.

Protein Sequencing

Human uterine tissue was homogenized in immunoprecipitation buffer and cleared by centrifugation at 200 *g*. The supernatant was incubated with affinity-purified α9 anti-cytoplasmic peptide antiserum cross-linked to protein A-Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) overnight at 4°C. The beads were then washed extensively with immunoprecipitation buffer and heated to 95°C for 5 min in 2% SDS, 300 mM 2-mercaptoethanol, 80 mM Tris, pH 6.8. The supernatant was concentrated on a Centricon-10 Concentrator (Amicon, Beverly, MA), subjected to 7.5% SDS-PAGE, transferred to PVDF membrane (Bio Rad Labs. Hercules, CA) for 3 h at 50 V in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11/10% methanol, and quantified by staining with 0.1% Coomassie blue R-250 in 40% methanol/1% acetic acid. A prominent band at 150 kD was microsequenced by the USCF Biomedical Resource Center (San Francisco, CA).

Results

Cloning and Sequencing of Human α9

A novel 223-nucleotide partial guinea pig integrin α subunit cDNA (αA) was previously reported by Erle et al. (13). To obtain the corresponding human sequence of the novel subunit, we used the previously described degenerate integrin α subunit forward primer (A14F: CGGAATTCGGIG-A(AG)CAG(AC)TIG(CG)I-(GT)CIGA(CT)TT(CT)GG), a degenerate reverse primer based on the αA sequence (AN2R: CAAGTCGACAA(AG)TGIGC(AG)TT(GC)TA-IGCIC(GT)(AG)TC), and cDNA made from human lung tissue. A band of the predicted size (204 nucleotides) was obtained (data not shown), subcloned into pBluescript, and sequenced. The resulting nucleotide sequence was 88% identical to the sequence of guinea pig αA and 39–54% identical to other known human integrin α subunits. For reasons discussed later, we have named this novel human integrin subunit α9.

The 204-nucleotide α9 cDNA fragment was used to screen a human lung cDNA library. One hybridizing clone, L1 (Fig. 1), was identified, purified, subcloned and sequenced. This 1,678-bp clone contained a 1,123-nucleotide open reading frame that included the probe sequence.

We were able to amplify α9 from cDNA from leukocyte cell lines U937 (clones U1–U3) and HL-60 (H1) by PCR using α9-specific primers. To isolate additional sequence from these cell lines, we used a previously described 3′-sequence extension technique (15) which employs PCR. One clone from HL-60 cDNA, H2, and one clone from U937 cDNA, U4, obtained by this method contained 117 nucleotides of α9 sequence.

PCR was performed on amplified purified human lung cDNA library DNA using α9-specific forward oligonucleotide primers and λgt11-specific primers (λgt11F and λgt11R). Individual bands were isolated, reamplified, subcloned, and sequenced. One clone, L2a, consisted of 300 nucleotides of α9 sequence. Clone L2a was used as a probe for screening the human lung library and one hybridizing clone, L2b, was isolated, purified, subcloned, and sequenced. Both clone L2a and clone L2b ended at nucleotide 1817, suggesting that clones L2a and L2b represented the same library clone.

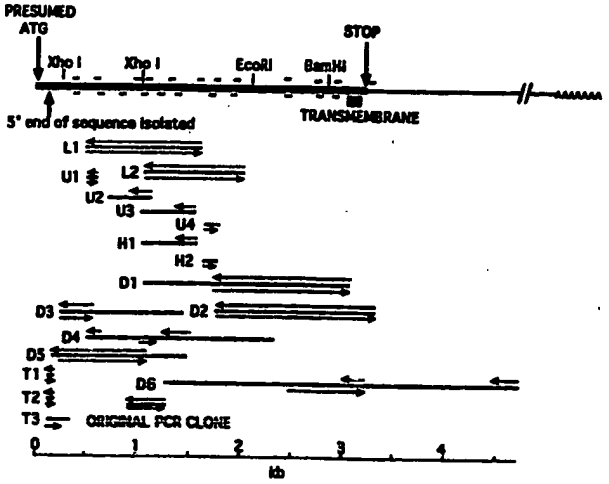


Figure 1. Map of α9 sequencing strategy. Shown are the location of clones used to obtain the partial sequence of human α9. Internal sequence was obtained by use of specific α9 sequencing primers (*dashes*) and by generating restriction fragments at sites shown. The clone labeled *original PCR clone* was obtained from human lung cDNA using homology based PCR. Clones labeled *L1* and *L2* were obtained from a human lung cDNA library. Clones labeled *U1–U4* were obtained by PCR from U937 cDNA. Clones *H1* and *H2* were obtained by PCR from HL-60 cDNA. Clones labeled *D1–D6* were obtained from a human small intestine cDNA library. Clones labeled *T1–T3* were obtained by PCR from Tera-2 cDNA. The direction and extent of sequencing are indicated with arrows. All reported sequence was sequenced completely in both directions from at least two independent clones. Noncoding sequences found on the 5′ end of clones *L1* (555 bp), D3 (108 bp), and *D6* (~1200 bp) are not indicated on the map.

We screened a human duodenal cDNA library with probes from clones L1 and L2. 12 independent hybridizing clones were identified, isolated, subcloned, and analyzed. Six of these were determined to contain additional α9 cDNA and were sequenced (D1–D6). D2 and D6 contained a predicted transmembrane domain, cytoplasmic domain, and translation stop codon. D6 also had an additional 1.1 kb, 3′ of the stop codon, which did not extend to the poly(A) tail.

To complete the α9 sequence, α9 protein was purified from human uterus using anti-α9 antiserum, described later, and microsequenced. The amino terminal sequence was equivocally determined to be YNLD(T/P)(Q/E). A series of degenerate forward oligonucleotide PCR primers were designed based upon these possible amino-terminal sequences. PCR amplifications were performed using each of the degenerate forward primers paired with α9-specific reverse oligonucleotide primers and Tera-2 cDNA (data not shown). Reactions using a degenerate forward primer based upon the sequence YNLDPQ and two different α9-specific reverse primers resulted in intense bands which were cloned and sequenced (T1–T3).

We have sequenced 3,139 nucleotides (Fig. 2) of α9 cDNA that contains a 3,000-nucleotide open reading frame that includes a termination signal but lacks an initiation codon. Based on this cDNA sequence and the amino terminal sequence we obtained, we predict the mature α9 protein to be 1,006 amino acids in length with a 947-amino acid extracellular domain, a 26-amino acid transmembrane domain, and

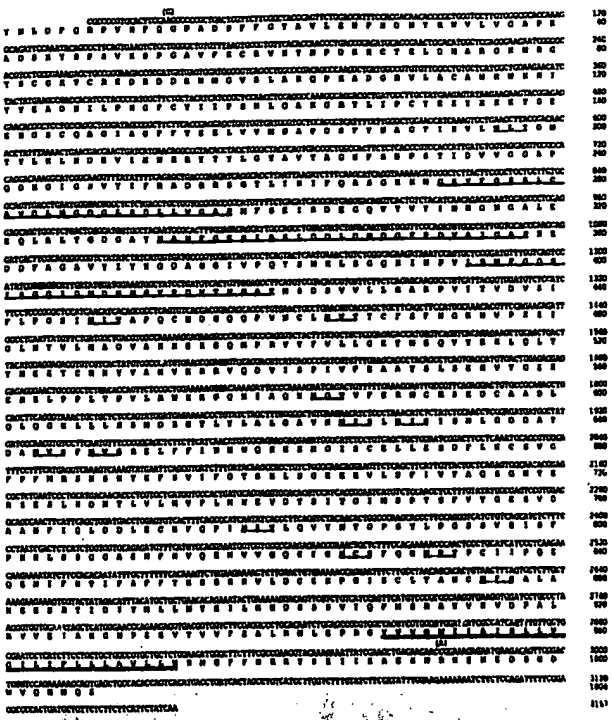


Figure 2. Human integrin α9 cDNA sequence and deduced amino acid sequence. The amino acid sequence is shown in one-letter code below the first nucleotide of each codon. The putative transmembrane domain is shown by a double underline. Asparagine residues (*N*) that are potential sites for N-glycosylation are indicated by dotted underline. Conserved putative metal binding domain are indicated by a single underline. Nucleotide 36 was determined to be A from some clones and G from others (indicated by a *G* in parentheses above the nucleotide location). Nucleotide 2974 was C in some clones and A in others. None of these nucleotide variations changed the deduced amino acid sequence. This sequence data is available from EMBL/GenBank/DDBJ under accession number L24158.

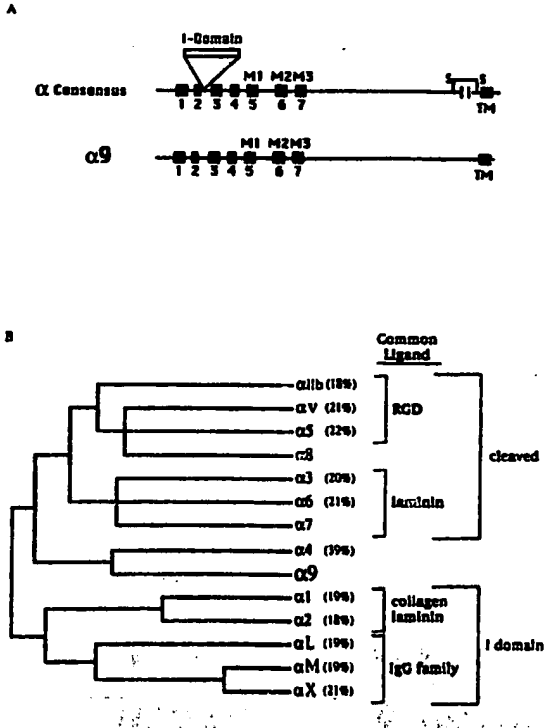


Figure 3. Comparison of α9 to other human integrin α subunits. (*A*) The predicted structure of α9 is shown schematically compared to the consensus structure of α subunits, with the NH_2-termini on the left. Conserved sequence repeats are indicated by black boxes numbered 1-7. The three conserved repeats that represent complete putative metal binding domains are labeled *M1-M3*. The I domain present in some subunits is indicated between conserved repeats 2 and 3. The cleavage site present in some subunits is indicated near the transmembrane domain by *S-S*. The transmembrane domain is indicated with *TM*. (*B*) The sequence similarity between all the previously reported human integrin α subunits is indicated by tree diagram (reference 21). The percent amino acid identity between α9 and each human integrin α subunit for which the sequence has been published in indicated parenthetically next to that subunit. Three subfamilies can be differentiated based on structural and functional features that correlate with sequence homologies: (*a*) subunits which are cleaved into two disulfide-linked fragments are involved in binding to the long arm of laminin or binding to RGD sequences present in certain extracellular matrix proteins, (*b*) subunits which contain the I domain are involved in binding to collagen and the cross region of laminin or to IgG superfamily members, and (*c*) other subunits (α4 and α9) which neither exhibit the conserved cleavage pattern nor contain the I domain.

a 33-amino acid cytoplasmic domain. α9 has 12 asparagine residues that are potential N-glycosylation sites (NXT/S).

The Relationship of α9 to Other Integrin α Subunits

Comparison of α9 to the general structure of integrin α subunits is indicated in Fig. 3 *A*. The consensus structure includes: a large NH_2 terminal extracellular domain containing seven conserved repeats (four partial and three complete putative metal binding domains) and in some cases the I domain, an insertion of approximately 200 amino acids, a single transmembrane-spanning domain, and a short COOH-terminal cytoplasmic domain. Some subunits are cleaved into two disulfide-linked fragments near the transmembrane domain (18). α9 contains the seven conserved repeats found in all known integrin α subunits. α9 does not contain an I domain nor is it cleaved. The cytoplasmic domain of α9, as with all α subunits, contains the highly conserved sequence GFF(R/K)R.

Sequence, structural, and functional relationships between the integrin α subunits are shown in Fig. 3 *B*. The integrin α subunit family has three distinct subfamilies. The first subfamily consists of the seven α subunits that undergo cleavage near the transmembrane domain. The second subfamily includes the five α subunits that contain an I domain. The third subfamily consists of α4, which contains neither the I domain nor undergoes cleavage yielding disulfide-linked fragments. The deduced partial amino acid sequence of α9 is 39% identical to the integrin α4 subunit sequence and 18–22% identical to the other known human integrin α subunit sequences. Based on its sequence and structural similarity t α4, α9 is clearly a member of the third integrin α subunit subfamily.

Fig. 4 shows the alignment of the predicted α9 amino acid sequence with α4. All 23 cysteine residues found in α9 align with those found in α4 (α4 contains one additional cysteine),

Figure 4. Alignment of α9 with its closest integrin α subunit relative, α4. The deduced amino acid sequences of human α9 and guinea pig αA (*reference* 13) and the previously reported sequence of the mature form of α4 (*reference* 46) are shown aligned using the one-letter amino acid code. Cysteine residues conserved between α9 and α4 are indicated by an asterisk (★). The conserved metal binding repeats common to all integrin α subunits are underlined. The predicted transmembrane domains are indicated by double underline. The cleavage site in α4 (++), between Arg558 and Ser559, is not present in α9.

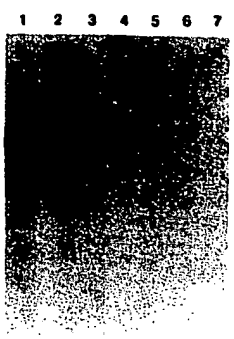


Figure 5. Identification of α9 mRNA by Northern blot analysis. Total RNA (30–40 μg/lane) isolated from human cell lines Caco-2 (lane *1*), Tera-2 (lane 2), HeLa (lane *3*), Raji (lane *4*), HuT 78 (lane *5*), MOLT-4 (lane *6*), and HL-60 (lane *7*) was probed for α9 mRNA using 2.1 kb of ^{32}P-labelled α9 cDNA. The positi ns of the 28- and 18-S ribosomal bands are indicated on the left.

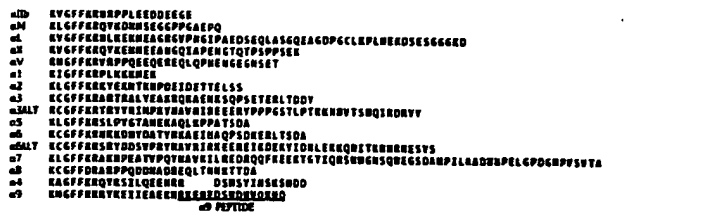


Figure 6. Alignment of integrin α subunit cytoplasmic domains. The COOH-terminal cytoplasmic sequences of the known human integrin α subunits are shown (*references* 2, 5, 9, 10, 28, 33, 39, 42–45). The amino acid sequence of the peptide used for production of α9-specific rabbit antiserum is underlined and labeled *α9 Peptide.*

including the 19 cysteines generally found in integrin α subunits. α4 contains a cleavage site following Lys557-Arg558 which is absent in α9.

α9 Is Expressed by Teratoma and Colon Carcinoma Cells and Forms a Heterodimer with β1

To demonstrate expression of α9 mRNA and to identify cell lines that express α9, Northern blots were performed. Total RNA was isolated from a variety of cell lines and analyzed by Northern blotting with a 2.1-kb α9 cDNA probe (Fig. 5). This probe hybridized with an ~7.0-kb mRNA band (size markers not shown) that was present in two of the cell lines tested, i.e., Tera-2 and Caco-2 cells (lanes *1* and 2). The smaller band may represent partially degraded α9 mRNA. Alternatively, a second α9 mRNA species derived by alternative splicing or an alternative polyadenylation signal may be present in these cells. Tera-2 cells are derived from an embryonal carcinoma, and Caco-2 cells from a colon carcinoma. The carcinoma-derived cell line HeLa (lane *3*) and the leukocyte-derived cell lines Raji, HuT 78, MOLT-4, and HL-60 (lanes *4–7*) were negative for expression of α9, as were cell lines U937 (histiocytic lymphoma), K-562 (chronic myelogenous leukemia), Daudi (Burkitt's lymphoma), AN3CA (endometrial carcinoma), and JEG-3 (choriocarcinoma) (data not shown).

The sequences of the cytoplasmic domains of the known vertebrate integrin α subunits are shown in Fig. 6. It is evident that the α9 cytoplasmic domain is distinct from all other subunits, except for very limited similarity to the α4 cytoplasmic domain. A 15-amino acid α9 peptide (underlined in Fig. 6) with an additional cysteine residue at the NH_2 terminus was used to generate polyclonal rabbit antiserum which was subsequently affinity purified on the same peptide coupled to Sepharose. The sequence of this peptide is distinct from the cytoplasmic domain sequences of the other α subunits, except for a three-amino acid identity (DSW) with α4. Cross-reactivity of the anti-α9 peptide serum with α4 or any other known α subunit is unlikely.

Immunoprecipitations were performed on surface labeled Tera-2 lysate with α9 antiserum or with a MAb against the integrin β1 subunit. The immunoprecipitates were analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 7). Immunoprecipitation with anti-β1 yielded major

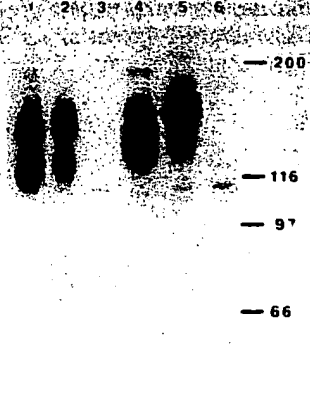


Figure 7. Immunoprecipitation of α9- and β1-containing integrins from lysates of Tera-2 cells. Aliquots of ^{125}I-surface-labeled lysates from Tera-2 cells were immunoprecipitated with a monoclonal anti-β1 antibody (lanes *1* and *4*), affinity purified polyclonal antiserum raised against a portion of the cytoplasmic domain of α9 (lanes 2 and *5*), and preimmune serum (lanes *3* and *6*). The proteins were analyzed by SDS-PAGE under n nreducing (lanes *1–3*) and reducing (lanes *4–6*) conditions followed by autoradiography. The positions of molecular size markers (in kD) are shown t the right. 10-fold less lysate was used in the β1 immunoprecipitation than in the α9 r preimmune serum immunoprecipitations.

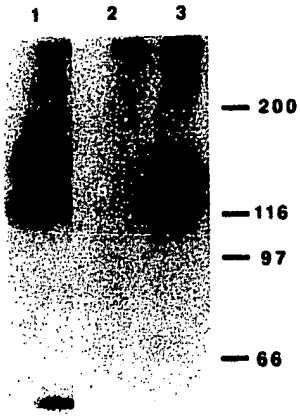


Figure 8. Immunodepleti n of β1 containing integrins. Aliquots of ^{125}I-surface-labeled lysates from Tera-2 cells were subjected to four rounds of immunoprecipitation with an anti-β1 monocl nal antibody or an anti-β6 mAb cross-linked to protein A-Sepharose. The resulting supernatants were immunoprecipitated with affinity purified α9 polyclonal antiserum. The immunoprecipitated material (anti-β1 first round immunoprecipitate, lane *1*; anti-β1 followed by α9 antiserum, lane *2*; anti-β6 followed by α9 antiserum, lane *3*) was analyzed by SDS-PAGE under nonreducing conditions followed by autoradiography. The positions of molecular size markers (in kD) are shown to the right.

nonreduced bands at 140 and 115 kD and a minor band at 180 kD (lane *1*). These bands changed upon reduction to 190, 150, and 130 kD (lane *4*). α9 antiserum immunoprecipitated a heterodimer consisting of 140 and 115 kD subunits nonreduced (lane 2) and 150 and 130 kD subunits reduced (lane *5*). The upper band migrated with the same apparent molecular mass as several other previously reported integrin α subunits (19). The lower band migrated with the same apparent molecular mass as β1 (19). The band present in α9 immunoprecipitations at 110 kD is also present in immunoprecipitations using preimmune serum (lanes *3* and *6*), and hence is nonspecific.

To determine if β1 was a β subunit partner for α9, Tera-2 lysates were subjected to four rounds of immunodepletion with either anti-β1 or anti-β6 antibody followed by immunoprecipitation with affinity purified α9 antiserum. The results (Fig. 8) show that α9 could not be immunoprecipitated from lysate depleted of β1 (lane *2*). Immunodepletion with the anti-β6 antibody (lane *3*) did not interfere with subsequent precipitation of α9, demonstrating that nonspecific loss of α9 does not occur during multiple rounds of immunodepletion. These results suggest that β1 is the predominant β subunit partner for α9 in Tera-2 cells. This justifies our terminology, following the convention of sequentially numbering the α subunit partners of β1.

α9 Is Widely Distributed in Normal Tissues

Because α9 was initially isolated from airway epithelial cell cDNA, we attempted to identify α9 in human airway tissue by immunohistochemistry. Fig. 9 *A*, shows a human airway biopsy section, including epithelium and submucosa, stained with hematoxylin. α9 antiserum intensely stained the airway epithelium (*B*). When the antiserum was incubated with α9 peptide prior to tissue staining, staining of the epithelium was substantially blocked (*C*).

Because we were able to detect α9 immunoreactivity in airway tissue, we examined the expression of α9 in other tissues. Secti ns of mouse tissue were stained with hematoxylin (Fig. 9, *D*, *G*, and *J*) α9 antiserum (*E*, *H*, and *K*), or α9 antiserum preblocked with α9 peptide (*F*, *I*, and *L*). In mouse esophagus (*D–F*), the basal layer of the epithelium (*arrow*) was stained by α9 antiserum. Skeletal muscle (*M*) present in this section also showed α9 immunoreactivity. In mouse small intestine (*G–I*) both the longitudinal and circumferential smooth muscle layers (*SM*) reacted with the α9 antiserum. Hepatocytes in the liver (*J*, *K*, and *L*) demonstrate cell surface localization of α9. A variety of other mouse tissues were analyzed for the presence of α9. Table I summarizes these results. The tracheal epithelium; basal epithelium of the larynx, pharynx, esophagus, skin, and cornea; smooth muscle of trachea, veins, duodenum, colon, stomach, and esophagus; skeletal muscle; hepatocytes; and splenic giant cells all demonstrated α9 immunoreactivity. In all of these cases, staining was blocked by incubation of the α9 antiserum with α9 peptide prior to tissue staining.

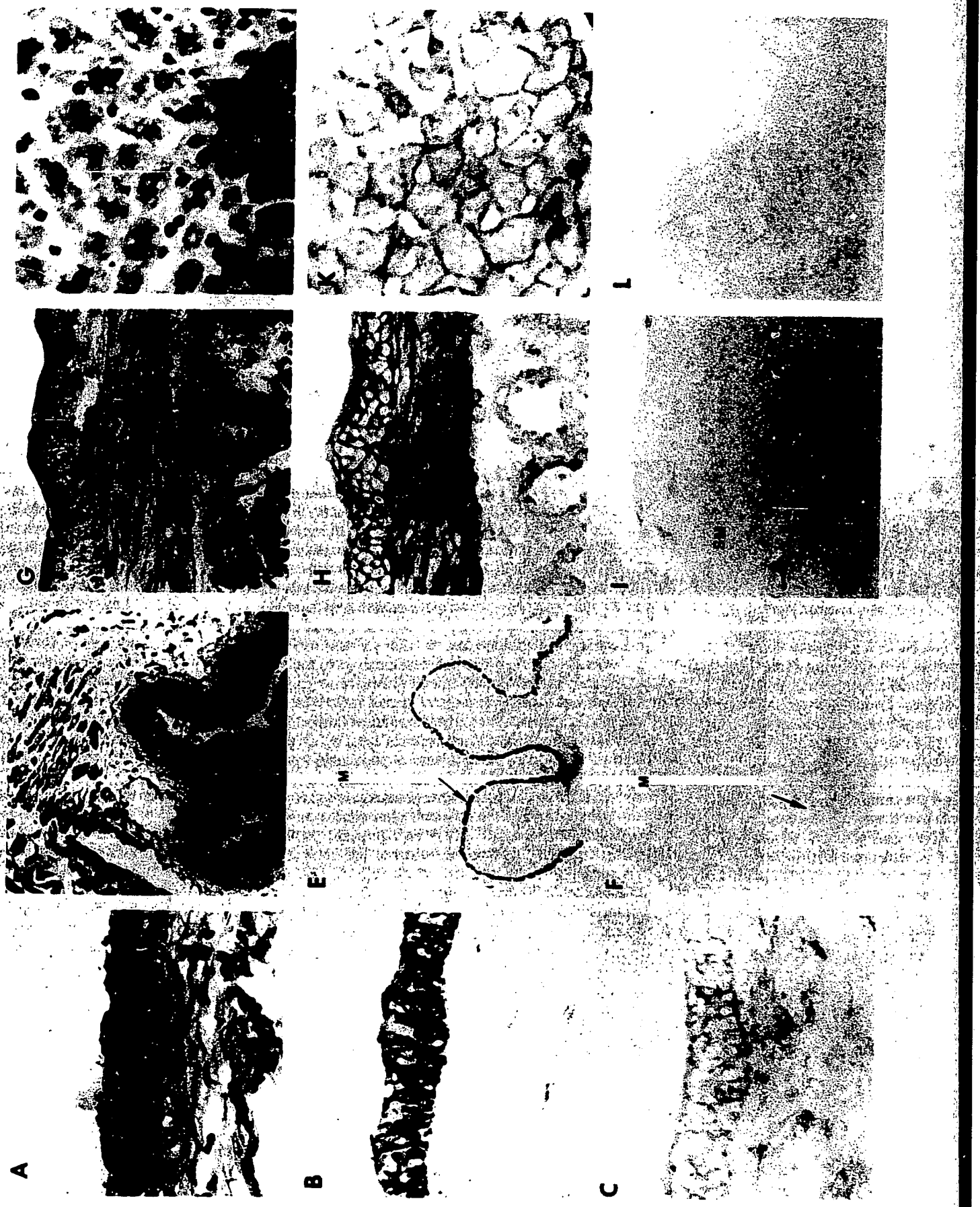
Discussion

This report presents three novel findings. First, we present the complete amino acid sequence, deduced from cDNA and amino terminal sequencing, of a new member of the human integrin α subunit family, α9. Second, we demonstrate that α9 forms an integrin heterodimer with the known β subunit, β1. Third, we show that α9 is expressed in a variety of cell types in vivo including airway epithelial cells, the basal layers of squamous epithelium, smooth muscle, skeletal muscle, and hepatocytes.

Comparison of the deduced amino acid sequence of α9 with the previously published sequences of human integrin α subunits clearly demonstrates that this protein is a member of the integrin α subunit family. α9 has high sequence homology with other human integrin α subunits and has predicted structural features common to the integrin α subunits including (*a*) a large extracellular domain containing four partial and three complete metal binding domains, 19 consensus cysteine residues, and several potential N-glycosylation sites; (*b*) a single transmembrane spanning domain; and (*c*) a short cytoplasmic domain containing the sequence GFF(K/R)R. Although α9 is the only human integrin α subunit to have the alternative sequence GFFRR, chicken α3 contains GFFRR (22). Both variations of this sequence are also found in the DNA binding domain of the members of the steroid hormone receptor superfamily (29). The functional significance of this sequence is not known, although it has been reported that a GFFKR-containing peptide can interact with the Ro/SS-A antigen (calreticulin) (35).

There are reports of four other integrin α subunits for which the human sequence has not been published. We do not believe that any of these four are identical to the subunit we describe based on the following evidence. The integrin subunits α7 and α8 have been cloned from other species, and

Figure 9. Immunohistochemical localization of α9. Frozen sections of human airway biopsies (*A–C*) and mouse esophagus (*D–F*), duodenum (*G–I*), and liver (*J–L*) were stained with hematoxylin (*A*, *D*, *G*, *J*), with α9 antiserum (*B*, *E*, *H*, *K*), or with α9 antiserum preincubated with α9 peptide (*C*, *F*, *I*, *L*). The α9 antiserum specifically stained airway epithelium, esophageal skeletal muscle (*M*) and basal cells of the squamous epithelium (*arrow*), duodenal smooth muscle (*SM*), and hepatocytes.


A
B
C
D
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F
G
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L
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M

Table I. Immunohistochemical Localization of α9 in Mouse Tissue

Tissue	α9 expression	Distribution
Trachea	+	Epithelium, smooth muscle
Larynx	+	Basal epithelium
Esophagus	+	Basal epithelium, smooth muscle
Skeletal muscle	+	
Stomach	+	Smooth muscle
Duodenum	+	Smooth muscle
Skin	+	Basal keratinocytes
Cornea	+	Basal epithelium
Veins	−	Smooth muscle
Aorta	−	
Pancreas	+	
Liver	−	Hepatocytes
Heart	+	
Spleen		Giant cells only (rare)

the resulting sequences have less than 21% amino acid identity to α9 (4, 38). Partial cDNA sequences of the human homologs of α7 and α8 are clearly distinct from the α9 sequence (our unpublished observations). Both of the subunits of a novel integrin expressed on activated leukocytes, termed the leukocyte response integrin, have different electrophoretic mobilities than does α9 (16). The previously identified HML-1 antigen is an integrin composed of a novel α subunit, αIEL, paired with β7 (7, 25, 47). The HML-1 antigen has been detected in intraepithelial lymphocytes and a subset of lymphocytes located in the submucosal layer of the intestine (8), whereas α9 was not detected in either the intestinal epithelial layer or submucosa.

We screened several cell lines by Northern blotting for expression of α9 and identified two cell lines, Tera-2 and Caco-2, that expressed significant levels of α9 mRNA. Immunoprecipitation of lysates from these cell lines with α9 anti-peptide antiserum precipitated a heterodimer consisting of a larger subunit of similar size to many other α subunits and a smaller subunit that comigrated with β1 (Caco-2 data not shown). Immunodepletion of β1 from Tera-2 lysates removed all detectable α9 immunoreactivity, suggesting that β1 is the principal β subunit partner for α9 in these cells. This increases the number of α subunits known to associate with β1 to 10. Three of these α subunits (αv, α4, and α6) can also form heterodimers with other β subunits. We cannot exclude the possibility that α9 is expressed in low abundance with another β subunit in Tera-2 cells or that α9 has other β subunit partners in other cell lines or in vivo.

The closest known relative of the α9 subunit is the α4 subunit. α9 and α4 share 39% amino acid identity and are both equally divergent from the other known α subunits. Consistent with their similarity, α9, like α4, is not composed of two disulfide-linked fragments and does not contain an I domain. Integrin α subunits generally demonstrate low correlation of sequence similarity with tissue distribution. Therefore, even though α9 and α4 are closely related, we would not necessarily expect them t have similar expression patterns. α4 was first identified on leukocytes (19) and has more recently been shown to be expressed on endothelial cells (31) and in developing, but not adult, skeletal muscle (36). In contrast, α9 was detected in adult skeletal and smooth muscle and in a subset of epithelial cells and is not generally expressed in lymphocytes or in endothelium. Although α9 was detected by PCR in two leukocyte cell lines, it could not be detected by Northern blotting in these cells. The overwhelming majority of lymphocytes present in sections of mouse spleen did not stain with the α9 antibody. However, rare, splenic giant cells did demonstrate α9 immunoreactivity, suggesting that α9 may be expressed in at least some leukocytes in vivo.

Our results show widespread expression of α9 in vivo. In airway epithelium and the basal layer of squamous epithelium, α9 appears to be distributed at cell–cell borders as well as at cellular contacts with basement membrane. In smooth muscle, skeletal muscle, and hepatocytes, α9 appears to be uniformly distributed over the cell surface. In these tissues, α9 is expressed diffusely at sites of homotypic cell to cell contact in cells that are not actively involved in spreading, migration, or any other obvious dynamic interaction with the extracellular matrix. These results suggest that α9 may be involved in homotypic cell–cell interactions. Although integrins are not generally thought to be involved in homotypic cell–cell adhesion in non-leukocyte cells, localization of integrins to cell–cell contacts has been shown for α2β1 in bronchial epithelial cells (1), cultured keratinocytes (6), and endothelial cells (27); for α3β1 in bronchial epithelial cells (1), cultured keratinocytes (6), and a variety of transformed cell lines (24); and for α5β1 in endothelial cells (27). Furthermore, contacts between cultured keratinocytes have been shown to be disrupted by antibodies directed against α3β1 and β1 (6). Mediation of cellular cohesion by integrins could result from direct binding of integrins to counter-receptors present on adjacent cells or by neighboring cells jointly binding to extracellular matrix proteins deposited in the intercellular space.

The results of this study demonstrate the existence of a previously unrecognized member of the integrin family that is widely expressed in vivo in differentiated cells that are not actively involved in migration, proliferation, or heterotypic interactions with other cells. These data suggest that this integrin may function in some aspect of normal tissue cohesion or homeostasis. More definitive functional characterization will require identification of the ligand or ligands for this receptor and the development of reagents that specifically interfere with its function.

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